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RESEARCH PAPER

Effects of mechanical damage and herbivore wounding on H_2O_2 metabolism and antioxidant enzyme activities in hybrid poplar leaves

AN Yu, SHEN Ying-bai*, ZHANG Zhi-xiang

College of Biological Science and Biotechnology, Beijing Forestry University, Beijing 100083, P. R. China

Abstract: The changes of hydrogen peroxide (H_2O_2) metabolism and antioxidant enzyme activities in a hybrid poplar (*Populus simonii* ×*P. pyramidalis* 'Opera 8277') in response to mechanical damage (MD) and herbivore wounding (HW) were investigated to determine whether H_2O_2 could function as the secondary messenger in the signaling of systemic resistance. Results show that H_2O_2 was generated in wounded leaves through MD and HW treatments and systemically in unwounded leaves around the wounded leaves. The activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) were also enhanced. However, the H_2O_2 accumulation and antioxidant enzyme activities were inhibited in MD leaves through the pretreatment with DPI (which is a specific inhibitor of NADPH oxidase). The results of this study suggest that H_2O_2 could be systemically induced by MD and HW treatments, and H_2O_2 metabolism was closely related to the change in SOD, APX and CAT activities. A high level of antioxidant enzymes could decrease membrane lipid peroxidation levels and effectively induce plant defense responses.

Keywords: antioxidant enzymes; herbivore wound; induced resistance; mechanical damage; reactive oxygen species

Introduction

Generation of reactive oxygen species (ROS), such as the superoxide radical (O_2^-) and H_2O_2, is a common response to pathogen invasion (Bolwell et al. 1999; Fath et al. 2002). Similarly, ROS are also generated in plant tissues in response to mechanical damage (Orozco-Cárdenas and Ryan 1999) and herbivore wounding (Mithöfer et al. 2004; Leitner et al. 2005; Maffei et al. 2006). ROS have a dual role. At low concentrations ROS act as second messengers involved in cell signaling transduction, and at high concentrations they are part of the direct defense and may also lead to programmed cell death (Vandenabeele et al. 2003).

In order to prevent oxidation burst due to production of ROS, plants have evolved complex mechanisms for scavenging ROS, which include both low molecule weight antioxidants and antioxidant enzymes. Superoxide dismutase (SOD) catalyzes superoxide radicals to produce H_2O_2 (Bowler et al. 1992). The H_2O_2 is then scavenged by catalase (CAT) and ascorbate peroxidase (APX) into H_2O and O_2 (Blokhina et al. 2003).

H_2O_2 is relatively stable and able to penetrate the plasma membrane as an uncharged molecule; thus, it is considered to act as a signal molecule in plants in their pathogen defense reaction and wounding stress response (Mehdy 1994; Bowler and Fluhr 2000). Orozco-Cárdenas and Ray (1999) found that H_2O_2 was generated in response to wounding at wound sites and systemically in distal leaves. However, Costet (1999) found no significant change in H_2O_2 content in the systemic tissues of tobacco following *Phytophthora megasperma* infection. Previously, wound-induced production of H_2O_2 has been described in *Zea mays*, *Oryza sativa* and *Arabidopsis thaliana* (Guan et al. 2000; Chandru et al. 2003; Chang et al. 2004). However, these experiments are focused on herbaceous plants and there is limited evidence to show such a process in woody plants.

In the present study, we analyzed the H_2O_2 accumulation and the activities of ROS scavenging enzymes in leaves after mechanical damage (MD) and herbivore wounding (HW). The aim of our study was to investigate if the increase in H_2O_2 content was coincided with the changes in the activities of antioxidant enzymes in leaves after wounding, and to determine whether H_2O_2 could function as the secondary messenger in the signaling of systemic resistance.

Materials and methods

Plant materials and treatment

Populus simonii ×*P. pyramidalis* 'Opera 8277' saplings were cultivated in pots (25.0 cm × 25.0 cm) under natural conditions

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Biography: AN Yu (1982-), female, Postgraduate in College of Biological Sciences and Biotechnology, Beijing Forestry University, Beijing 100083, P. R. China. Email: anyu-02@163.com

*Corresponding author: SHEN Ying-bai, Email: ybshen@bjfu.edu.cn

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in March, 2007. They were watered daily and supplied with a nutrition solution (5% Hoagland) every two weeks.

Similar plants were selected for minimizing differences between plants. In mechanical damage experiment, the expanded leaves of poplar sapling were damaged by scratching the leaf with a punch, accounting for 20% of total leaf area. For herbivore wounding treatment, third instars larvae of *Clostera anachoreta* were not fed for 12 h before they were used in the experiment. The insect-fed leaves were obtained by placing 8−10 heads of *C. anachoreta* larvae on each plant for 30 min. To check the effect of the inhibitor, the leaves of poplar were sprayed with a 10-μM DPI solution (diphenylene iodonium chloride, which was a specific inhibitor of NADPH oxidase) for 12 h before the leaves were wounded. Intact poplar leaves in the same condition were used for control. After the treatments, the wounded leaves and the unwounded leaves around the wounded leaves were collected after 0.5, 1, 2, 4 and 8 h from different plants. These leaves were frozen immediately in liquid nitrogen and stored at -80°C for the determination of antioxidant enzyme activities and H_2O_2 content. All experiments were repeated three times.

Determination of hydrogen peroxide

H_2O_2 content was measured according to a procedure described by Liu et al. (2000). Leaves (0.2 g) were ground to a fine powder in liquid nitrogen and homogenized in 3-mL cold acetone. The homogenate was centrifuged at 12 000 g for 20 min at 4°C. The 2.5-mL extracting agent (CCl_4:$CHCl_3$ = 3:1, v/v) and 2.5-mL ddH_2O were added to 1-mL supernatant, blended and centrifuged it at 7 000 g for 10 min. The supernatant was used to measure the H_2O_2 content. The reaction mixture contained 1-mL supernatant and 10-μL CAT (30°C for 10 min), and the final concentration was 3 μg/mL. Blank mixture was obtained by adding 10-μL inactive CAT to 1-mL supernatant, then kept the reaction and blank mixtures at 25°C for 10 min. 1-mL of 0.2-M phosphate buffer (pH 5.9) and 1-mL Ti (IV) - PAR colorimetric reagent were added to the reaction and blank mixtures, and kept the mixtures in the dark for 1 h after a water-bath at 45°C for 20 min. Ti (IV) - PAR colorimetric reagent was made daily by mixing 1:1 (v/v) of 0.1-mM 4-(2-pyridylazo) resorcinol and 0.1-mM potassium titanium oxalate and maintained in ice condition. The absorbance of the reaction mixture was read at 508 nm. The H_2O_2 content was determined by comparing the absorbance against the standard curve of H_2O_2.

Enzymes assays

Determination of superoxide dismutase

SOD activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT) as described by Chen et al. (2007). Leaves (0.2 g) were ground to a fine powder in liquid nitrogen, and then 1.5-mL ice-cold phosphate buffer of 50-mM (pH 7.0, 1% PVP) was added. The homogenate was centrifuged at 10 000 g for 20 min at 4°C and the supernatant used as crude extract for SOD activity assays.

The reaction mixture (3-mL) contained 50-mM phosphate buffer (pH 7.8), 0.01-mM EDTA, 13-mM methionine, 75-μM NBT, 2-μM riboflavin and 50-μL enzyme extract. The glass tubes containing the mixtures were shaken and illuminated with two 20 -W fluorescent lamps for 20 min, and then the lights were switched off and the tubes were placed in the dark. Blank and control were run the same way but without illumination and enzyme, respectively. The absorbance of the reaction mixture was measured at 560 nm. One unit of SOD activity (U) was defined as the amount of enzyme required to cause a 50% inhibition of NBT photochemical reduction. The result was expressed as $U \cdot g^{-1}$ FW.

Determination of catalase and ascorbate peroxidase

Leaves (0.1 g) were ground to a fine powder in liquid nitrogen, and then added 1-mL ice-cold K-phosphate of 50-mM (pH 7.0, 0.1-mM Na_2EDTA). The homogenate was centrifuged at 10 000g for 20 min at 4°C and the supernatant was used as a crude extract for CAT and APX activity assays.

CAT activity was measured according to the protocol of Song et al. (2001). The reaction mixture consisted of 2-mL K-phosphate buffer of 50-mM (pH 7.0), 1-mL H_2O_2 of 50-mM and 30-μL enzyme extract. The reaction was started by adding the enzyme extract. The decrease of H_2O_2 was monitored at 240 nm. The result was expressed as $OD_{240} \cdot min^{-1} \cdot g^{-1}$ FW.

APX activity was assayed according to a method develop by Shen et al. (1996). The reaction mixture (3-mL) was composed of 50-mM K-phosphate buffer (pH 7.0), 0.1-mM EDTA, 0.3-mM ascorbate, 0.06-mM H_2O_2, and 0.1-mL enzyme extract. The reaction was started by adding H_2O_2 and determined by the decrease in absorbance at 290 nm due to ascorbic acid oxidation for 1 min. The activity was calculated using the extinction coefficient (2.8 $mM^{-1} \cdot cm^{-1}$) for ascorbate. The result was expressed as $U \cdot min^{-1} \cdot g^{-1}$ FW.

Statistical analysis

The basic data was arranged by EXCEL, and the results presented were the mean of three replicates. Mean was compared by ANOVA and Duncan's Multiple Range Test (P<0.05) by SPSS.

Results

Effect of different treatments on H_2O_2 content in treated leaves

We assayed the production of H_2O_2 in response to mechanical damage (MD) and herbivore wounding (HW) treatments as shown in Fig. 1. H_2O_2 content in HW leaves increased gradually with increasing treatment time and increased by 70.5% above the control at 2 h of treatment, then declined at 4 h. H_2O_2 content in MD leaves reached its maximum after 1 h and then dropped after 2 h, but the content increased slightly after 8 h. A multiple comparison test showed that H_2O_2 in both MD and HW leaves had a significant difference (P<0.05) from the control at 0.5 h, but no significant difference was found between control and MD leaves

from 1 h to 4 h. H_2O_2 content in HW leaves was significantly higher than that in control and MD leaves from 1 h to 4 h.

To confirm that the accumulation of H_2O_2 in the leaves was the result of the product of an oxidase, DPI was supplied to leaves for 12 h before mechanical damage was inflicted. The H_2O_2 accumulation in MD leaves through the pretreatment with DPI was inhibited by 15.9% compared to unwounded leaves through the pretreatment with DPI after 0.5 h, but there was no significant difference between them (Fig. 1).

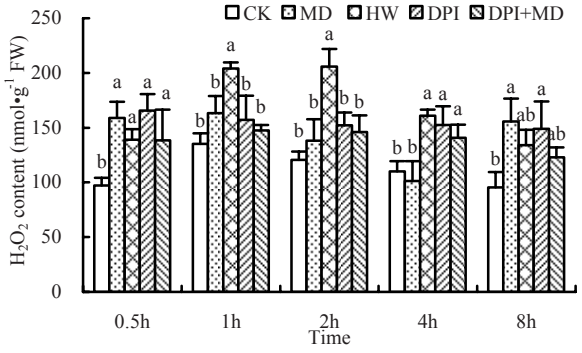

Fig. 1 Changes of H_2O_2 content in mechanical damage (MD) and herbivore wounding (HW) leaves of hybrid poplar

DPI treatment (which is a specific inhibitor of NADPH oxidase). Values are expressed as mean ± SE (n=3). Different letters above bars indicate significant differences among the different treatments at P<0.05 level.

Effect of different treatments on activities of antioxidant enzymes

The changes of antioxidant enzyme activities from MD and HW treatments on the sampling leaves were shown in Fig. 2. There is a significant increase in SOD activity in both MD and HW leaves. Compared with control at 0.5 h (P<0.05), SOD activity increased by 3.0% and 4.3% above the control. But there was no significantly difference in SOD activity between the HW leaves and the control after 1 h (Fig. 2A). With the increase in treatment time, SOD activity reached a peak at 1 h after mechanical damage and dropped to its original level at 8 h. A significant increase in SOD activity was observed in MD leaves, compared with control and HW leaves from 1 h to 4 h.

It was found that APX activity significantly (P<0.05) increased in both HW and MD leaves, compared with control, but there was no significantly difference in APX activity between the HW leaves and the control at 1 h and 4 h (Fig. 2B). Also the APX activity shows that there are significant differences between MD and HW leaves after treatment. The increase in APX might be due to an increase in H_2O_2 production during the treatment.

The activity of CAT significantly increased (P<0.05) in MD leaves compared with control and the highest activity increased by 76.2% over the control at 2 h. CAT activity in HW leaves was significantly lower than that in the MD leaves, increased by 130% and 17.8% at 0.5 h and 2 h, compared with the control, respectively (Fig. 2C).

A decrease in the activities of antioxidant enzymes was observed in the MD leaves through the pretreatment with DPI, compared to unwounded leaves through the pretreatment with DPI (Fig. 2). A significant decrease (P<0.05) in CAT activity was observed between MD leaves and unwounded leaves through the pretreatment with DPI. It is likely a consequence of inhibition of ROS production (including H_2O_2) resulting from the inactivation of NADPH oxidase by DPI.

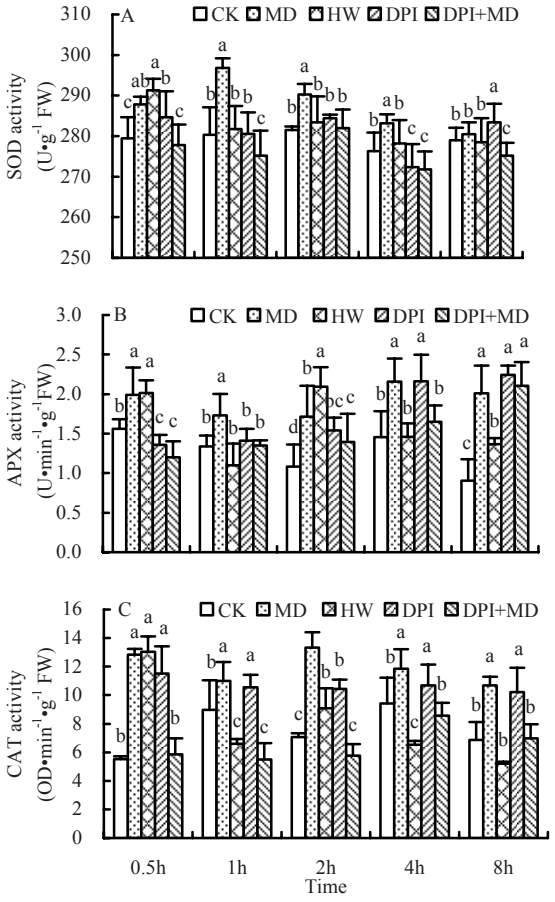

Fig. 2 Changes of antioxidant enzyme activities in MD and HW leaves of hybrid poplar

(A) SOD activity, (B) APX activity and (C) CAT activity with mechanical damage (MD) and herbivore wound (HW) treatment. Values are expressed as mean ± SE (n=3). Different letters above bars indicates significant differences among the different treatment at P<0.05 level.

Changes of H_2O_2 content in wounded and unwounded leaves

The result showed that H_2O_2 content increased not only in the MD leaves but also in the U-MD and D-MD leaves after mechanical damage (Fig. 3A). Initially, the H_2O_2 content was higher in U-MD leaves than that in D-MD leaves, but after 4 h the H_2O_2 content in U-MD was lower than that in D-MD leaves. A significant difference in H_2O_2 content was observed in U-MD and D-MD leaves, compared with control except 1 h and 2 h after treatment.

H_2O_2 content in HW leaves increased gradually with increasing treatment time, and reached a peak at 2 h and then decreased

(Fig. 3B). The changes of H_2O_2 content in U-HW and D-HW leaves were less than that in HW leaves, and significantly ($P<0.05$) different from the control except 0.5 h after treatment. The trend of H_2O_2 in unwounded leaves after HW treatment was similar with that in the unwounded leaves after MD treatment.

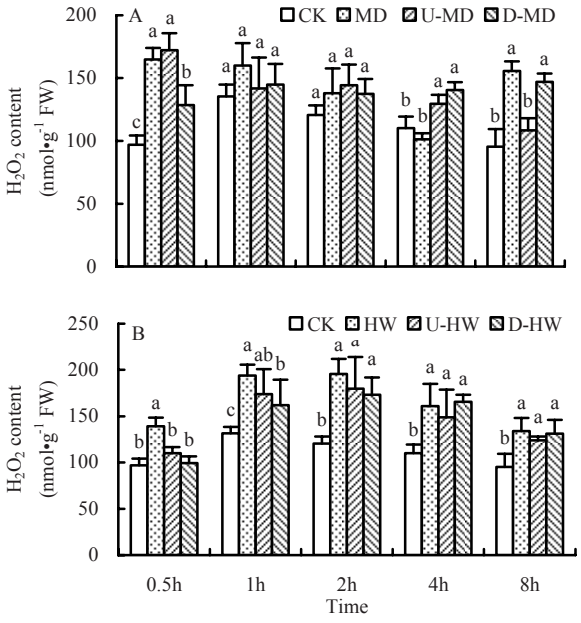

Fig. 3 Changes of H_2O_2 content in different leaves of hybrid poplar

(A) Mechanical damage treatment: (MD) mechanical damage leaves, (U-DW) the leaves above the MD leaves; (D-MD) the leaves below the MD leaves. (B) Herbivore wound treatment: (HW) herbivore wound leaves; (U-HW) the treated leaves above the HW leaves and (D-HW) the leaves below the HW leaves. Values are expressed as mean ± SE (n=3). Different letters indicates significant differences at $P<0.05$ level.

Discussion

In higher plants under abiotic and biotic stress conditions, an overproduction of ROS takes place, which act as mediators of oxidative damage (Dat et al. 2000; del Río et al. 2002). The generation of ROS, which include H_2O_2, is required for function of oxidative cross-linking of cell wall components and for the activation of many genes implicated in plant defense (Somssich and Hahlbrock 1998).

In order to obtain a better understanding of the production of an oxidative burst after MD and HW treatment, we monitored the early production of H_2O_2 in relation to the activity of SOD, APX and CAT in poplar leaves.

In response to herbivore wounding, H_2O_2 content were higher, compared to mechanical damaged leaves (Fig. 1). H_2O_2 content in HW leaves increased gradually with increasing treatment time and increased by 70.5% above the control at 2 h, and declined at 4 h. The H_2O_2 content in MD leaves reached its maximum after 1 h and dropped after 2 h. The different changes of H_2O_2 content between HW and MD treatment as the results of regurgitate of herbivores. Because mechanical damage only partially mimics the response of plants to herbivore feeding. Orozco-Cárdenas and Ray (1999) found that H_2O_2 generated in response to damage can be detected within 1 h after damage. ROS also have been associated with plant herbivore interactions (Mithöfer et al. 2004; Leitner et al. 2005). De Vos et al. (2006) demonstrated that herbivore-induced resistance against insect feeding could not be mimicked by mechanical damage alone in *Arabidopsis*.

NADPH oxidase is thought to generate superoxide anion ($O_2^{\cdot-}$) at the plasma membrane (O'Donel et al. 1993), and then is converted to H_2O_2 through the action of SOD (del Río et al. 2002). The H_2O_2 accumulation in MD leaves through the pretreatment with DPI was decreased after 0.5h, compared to unwounded leaves treated through the pretreatment with DPI (Fig 1). It indicated that the pretreatment with DPI inhibited the production of H_2O_2 induced by wounding, suggesting that the involvement of NADPH oxidase contributed significantly to H_2O_2 production. NADPH oxidase has been implicated in the production of ROS during the defense responses of plants against pathogens and herbivores attacks (Doke et al. 1996; Lamb and Dixon 1997; Orozco-Cárdenas and Ryan 1999), and also found to be induced by mechanical damage (Bochkov et al. 2002).

In order to prevent oxidation burst due to production of ROS, several antioxidant enzymes are responsible for scavenging of ROS. SOD is a major scavenger, which converts the $O_2^{\cdot-}$ to O_2 and H_2O_2 (Bowler et al. 1992). In the present study, the activity of SOD significantly increased in both HW and MD leaves, compared with control (Fig. 2A). The increasing of SOD activity was correlated with the generation of H_2O_2 in wounded leaves (Fig. 1), which proved to have greater $O_2^{\cdot-}$-scavenging ability. H_2O_2 is expected to be responsible for membrane lipid peroxidation, so plants need to scavenge it by CAT and APX into H_2O and O_2 (Blokhina et al. 2003; Sairam et al. 2005). Our results showed that APX activity was significantly increased in both HW and MD leaves after treatment (Fig. 2B), but dropped in HW leaves after 1 h. Also there is a significant difference in APX activity between MD and HW leaves. This is more likely due to the harmful effect of over-production of H_2O_2. The activity of CAT increased in response to two treatments. In MD leaves, the activity reached a peak at 2 h. In HW leaves, the increase was less than that in the MD leaves after treatment (Fig. 2C). It can be suggested that H_2O_2 induced by MD is successfully detoxified by CAT. These two enzymes, conjunction with SOD, play an essential protective role in the scavenging process and constitute the major defense system against ROS in plants.

Our results show that H_2O_2 content increased not only in MD leaves but also in U-MD and D-MD leaves after mechanical damage (Fig. 3A). The changes of H_2O_2 content in U-HW and D-HW leaves were similar and less than that in HW leaves (Fig. 3B). It suggests that H_2O_2 may function as diffusible signaling molecule inducing defenses in woody plants. Orozco-Cárdenas and Ray (1999) found that H_2O_2 has been generated in response to wounding at wound sites and systemically in distal leaves, indicating that the generation of H_2O_2 might be regulated by a systemic signaling system.

Responses of plant tissues to mechanical injury have intensively been study field in the context of attacks by herbivore and pathogen-related responses, e.g. systemic acquired resistance

(SAR) (Paul et al. 2000; León et al. 2001; Bostock et al. 2001). Furthermore, the information described here extends earlier observations that oxidative stress as well as an antioxidant defense system occurs in MD and HW treatments. The elevated H_2O_2 content also may potentiate the plants' defense responses against invading pathogens, in which ROS play an important role. The change of H_2O_2 content and the activities of the antioxidant enzymes SOD, APX and CAT in wounded and unwounded leaves had a potential important physiological role in mechanical damage and herbivore wounding stress. Understanding the complexity of the physiological responses in this process is a major challenge for future research.

It remains unresolved in this study that how the increase of ROS concentration is perceived and translated into signals. Further research addressing the above questions may help with elucidating the mechanisms of active resistance of woody plants in preventing pathogen and herbivores invasion.

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